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(54) Method for producing L-amino acid by fermentation

(57) L-amino acid such as L-threonine and L-glutamic acid is produced by cultivating a bacterium belonging to the genus *Escherichia* which is transformed with a gene coding for pyruvate carboxylase and has L-amino acid productivity, producing and accumulating L-amino acid in the medium and collecting the L-amino acid from the medium.

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Description

Technical Field

[0001] The present invention relates to a method for producing L-amino acid, specifically relates to a method for producing L-threonine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline or L-isoleucine using a bacterium belonging to the genus Escherichia.

Background Art

[0002] L-amino acids such as L-threonine and L-glutamic acid have conventionally been produced by fermentation methods principally utilizing coryneform bacteria which belong to the genera *Brevibacterium, Corynebacterium,* and *Microbacterium* or mutants thereof ("Amino Acid Fermentation", Gakkai Shuppan Center, pp.195-215, 1986).

[0003] On the other hand, there has been disclosed techniques for breeding of L-amino acid-producing bacteria belonging to the genus *Escherichia* utilizing genetic recombination techniques.

[0004] For example, there has been disclosed a method of producing L-lysine using *Escherichia coli* in which genes coding for dihydrodipicolinate synthetase, aspartokinase are desensitized to feedback inhibition by L-lysine and L-threonine, and diaminopimelate dehydrogenase (or tetrahydrodipicolinate succinylase and succinyldiaminopimelate deacylase) are enhanced (WO 95/16042).

[0005] Though the productivity of L-amino acids has considerably been improved by breeding of such microorganisms as mentioned above or production processes have been improved, it is still desired to develop more efficient processes for producing L-amino acids in order to meet the expected markedly increased future demand of the amino acids.

[0006] Pyruvate carboxylase (pyruvate:carbon dioxide ligase (ADP-forming), EC 6.4.1.1] is a biotine containing enzyme. It catalyses the carboxylation of pyruvate to form oxaloacetate. Pyruvate carboxylase has not been known in *Eschericiha coli*, but *Bacillus subtilis* (Diesterhaft, M.D. and Freese, E., J. Biol. Chem., 248, No.17, 6062-6070 (1973)) and *Corynebacterium glutamicum* (Peters-Wendisch, P. G. et al., *Microbiology, 144*, 915-927 (1998)) have pyruvate carboxylase and the nucleotide sequences of the genes coding for the enzymes have been reported.

[0007] Further, there has been known *Corynebacterium glutamicum* in which an activity of pyruvate carboxylase is enhanced or *Corynebacterium glutamicum* of which pyruvate carboxylase gene is inactivated (Peters-Wendisch, P. G. et al., Microbiology, 144, 915-927 (1998)). However, these microorganisms were created as experimental materials to study enzymes necessary for growth with glucose. The relation between the activity of pyruvate carboxylase and productivity of L-amino acids has not been known. Also, there has not been known an attempt to introduce the pyruvate carboxylase gene into a bacterium belonging to the genus *Escherichia*.

Summary of the Invention

[0008] The present invention has been accomplished from the aforementioned point of view, and its object is to provide a method for producing L-amino acid, especially L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline or L-isoleucine in high efficiency.

[0009] As a result of diligent investigation in order to achieve the object described above, the present inventors have found that introduction of a gene coding for pyruvate carboxylase (hereinafter referred to as "*pyc* gene") into a bacterium belonging to the genus *Escherichia* can improve L-amino acid productivity of the bacterium. Thus, the present invention has been completed.

[0010] That is, the present invention is a bacterium belonging to the genus *Escherichia* which is transformed with a gene coding for pyruvate carboxylase and has L-amino acid productivity.

[0011] The present invention also provides the bacterium described above wherein the gene coding for pyruvate carboxylase is derived from a bacterium belonging to the genus *Bacillus*.

[0012] The present invention further provides the bacterium described above wherein the gene coding for pyruvate carboxylase is introduced into said bacterium in a low copy number.

[0013] The present invention still further provides the bacterium described above wherein the L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine.

[0014] The present invention also provides a method for producing L-amino acid comprising cultivating the above bacterim in a medium, producing and accumulating the L-amino acid in the medium, and collecting the L-amino acid from the medium.

[0015] The present invention further provides the method described above wherein the L-amino acid is selected the group consisting of L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine.

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[0016] The term "L-amino acid procudtivity" means a property that a bacterium produces and accumulates the L-amino acid in a medium in a larger amount than a wild type strain thereof.

[0017] According to the present invention, productivity of L-amino acid such as L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline or L-isoleucine can be improved in a bacterium belonging to the genus *Escherichia*. Further, the present invention may be utilized to breeding of an L-amino acid producer belonging to the genus *Escherichia*.

Brief Description of the Drawings

[0018]

Figure 1 shows a scheme of constructing *Bacillus sutilis* pycA::KmR recipient strain for cloning *pycA* gene,
Figure 2 shows a scheme of cloning pycA gene from *Bacillus subtilis* 168, and
Figure 3 shows a scheme of construction of the plasmid containing *pycA* gene.

Detailed Description of the Invention

[0019] The present invention will be explained in detail below.

(1) A bacterium belonging to the genus *Escherichia* transformed with *pyc* gene

[0020] A bacterium belonging to the genus *Escherichia* of the present invention is a bacterium transformed with pyc gene. As the bacterium belonging to the genus *Escherichia*, there exemplified by *Escherichia coli*. While the *pyc* gene used in the present invention is not particularly limited so long as it can code a protein having pyruvate carboxylase activity, examples of the pyc gene include, for example, *pyc* gene derived from bacteria belonging to the genus *Bacillus* or pyc gene from coryneform bacteria. The nucleotide sequences of the *pyc* gene derived from *Bacillus subtilis* (Genbank/EMBL/DDBJ Accession Z97025, NID g2224758) and *Corynebacterium glutamicum* (Peters-Wendisch, P. G. et al., Microbiology, 144, 915-927 (1998)) has been reported. Therefore, *pyc* gene can be obtained by PCR (polymerase chain reaction: White,T.J. et al., Trends Genet., 5,185 (1989)) using primers synthesized according to the nucleotide sequences from chromosomal DNA of bacteria belonging to the genus *Bacillus* such as *Bacillus subtilis* or coryneform bacteria such as Corynebacterium glutamicum as a template.

[0021] A transformant of Escherichia coli 44/pMW119-pycA, which harbors pMW119-pycA containing pyc gene of *Bacillus subtilis* has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depositary, GNIIgenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, since August 30, 1999 under a Registration number of VKPM B-7822, and transferred from the original deposit to international deposit based on Budapest Treaty on September 1, 2000.

[0022] A transformant of Escherichia coli MG442/pMW119-pycA, which harbors pMW119-pycA containing pyc gene of *Bacillus subtilis* has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depositary, GNIIgenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, since August 30, 1999 under a Registration number of VKPM B-7821, and transferred from the original deposit to international deposit based on Budapest Treaty on September 1, 2000.

[0023] Incidentily, the gene coding pyruvate carboxylase of *Bacillus subtilis* (*pycA* gene) has been cloned as described below in a process of accomplishing the present invention.

[0024] First, a *pycA* gene-deficient strain of *Bacillus subtilis* was constructed. Then, using the strain as a recipient, *pycA* gene was cloned by isolating a DNA fragment which complemented citrate or aspartate auxotrophy of the strain from a genomic DNA library of a wild type strain of *Bacillus subtilis*. Since *Bacillus subtilis* forms oxaloacetate by pyruvate carboxylase, *Bacillus subtilis* cannot grow on minimal medium if it is deficient in the enzyme. However the growth is repaired by addition of L-aspartate, L-glutamate, citrate or succinate. The nucleotide sequence of *pycA* gene and the amino acid sequence coded by the gene are shown in SEQ ID Nos: 3 and 4.

[0025] The *pycA* gene-deficient strain is obtained, for example, as described below. A partial DNA frament of *ylaP* gene located immediately after the *pycA* gene on the chromosome is obtained by PCR. As primers for PCR are exemplified by oligonucleotides of SEQ ID NOs:1 and 2. Then a wild type strain of *Bacillus subtilis* is transformed with a plasmid DNA containing the obtained DNA fragment and a marker gene, followed by integrating the plasmid DNA into *ylaP* gene on the chromosomal DNA by homologous recombination. Next, chromosomal DNA library of the obtained strain is constructed using a restriction enzyme which recognizes a restriction site inside of *pycA* gene, for exmaple, *PstI*. The recombinant plasmid containing the marker gene and a partial pycA gene fragment may be obtained by tranforming a wild type strain of *Bacillus subtilis* with the library and selecting clones expressing the marker gene. Then a wild type

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strain of *Bacillus subtilis* is transformed with the linearized recombinant plasmid and clones expressing the marker gene are selected to obtain *pycA* gene-deficient straind in which the plasmid DNA is integrated in *pycA* gene on chromosomal DNA of the strain.

[0026] Transformation of a bacterium belonging to the genus *Escherichia* with *pyc* gene may be performed by inserting *pyc* gene into an appropriate vector which functions in a bacterium belonging to the genus *Escherichia* to construct a recombinant vector, and introducing the recombinant vector to a bacterium belonging to the genus *Escherichia*.

[0027] The vector is exemplified by plasmid such as pBR322, pMW118, pMW119, pUC18, pUC19 or the like, phage vector such as λ1059, λBF101, M13mp9 or the like, and transposon such as Mu, Tn10, Tn5 or the like. However, in the present invention, a low copy plasmid such as pMW118 and pMW119 is preferable, because a transformant harboring a vector may be unstable if a high copy plasmid is used to introduce *pycA* gene as a vector.

[0028] The introduction of a DNA into a bacterium belonging to the genus *Escherichia* can be performed, for example, by a method of D. A. Morrison (Methods in Enzymology 68, 326 (1979)) or a method in which recipient bacterial cells are treated with calcium chloride to increase permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)) and the like.

[0029] Preparation of the genomic DNA, construction of genomic DNA library, hybridization, PCR, preparation of plasmid DNA, digestion and ligation of DNA, transformation and the like are performed according to the methods which are known to a person skilled in the art. Such methods were described by Sambrook, J., Fritsche, E. F., Maniatis, T. in Molecular Cloning, Cold Spring Harbor Laboratory Press, 1.21 (1989).

[0030] L-amino acid productivity of a bacterium belonging to the genus *Escherichia* can be improved by conferring or enhancing pyruvate carboxylase activity on the bacterium through transforming the bacterium with *pyc* gene. This is probably because oxaloacetic acid is formed not only from phosphoenolpyruvic acid but also from pyruvic acid. That is, transport of glucose (or sucrose) molecule to a bacterial cell which is mediated by phosphoenolpyruvic acid system proceeds by consuming one molecule of phosphoenolpyruvic acid being accompnied with formation of one molecule of pyruvic acid. Although formed pyruvic acid is not directly utilized for L-amino acid synthesis in a bacterial cell, L-amino acid productivity may be increased by formation fo oxaloacetic acid from pyruvic acid.

[0031] As a bacterium belonging to the genus *Escherichia* to which *pyc* gene is to be introduced, a strain having an ability to produce objective L-amino acid is used. Alternatively, a bacterium belonging to the genus *Escherichia* which is transformed with *pyc* gene may be conferred L-amino acid productivity. Incidentally, *pyc* gene of *Escherichia coli* has not been known, however, if the gene is found in *E. coli* hereafter, the gene may be used.

[0032] Examples of L-amino acid-producing bacteria belonging to the genus *Escherichia* are described below.

(1) L-threonine-producing bacteria

[0033] The L-threonine-producing bacteria belonging to the genus *Escherichia* may be exemplified by strain MG442 (deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depositary, GNIIgenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, under a Registration number of VKPM B-1628)(USP 4,278,765; Guayatiner M.M. et al., *Genetika* (in Russian), *14*, 947-956 (1978)). The strain MG442 had been obtained as a mutant resistant to L-threonine analogue, 2-amino-3-hydroxyvaleric acid, it was also an isoleucine auxotroph of leaky type. It produces L-threonine and L-glutamate as a by-product.

[0034] Alternatively, a transformant of MG442 which is transformed with pVIC40 (USP 5,175,107) may be preferably used as L-threonine-producing strain.

(2) L-Lysine-producing bacteria

[0035] The L-lysine-producing bacteria belonging to the genus *Escherichia* may be exemplified by various bacterium described in WO95/16042. The L-lysine-producing bacteria are concretely exmeplified by a bacterium in which aspartokinase activity and dihydrodipicolinate reductase activity are increased such as W3110(tyrA)RSFD80+pdapB.

(3) L-homoserine-producing bacteria

[0036] The L-homoserine producing bacteria belonging to the genus *Escherichia* may be exemplified by strain 44 (*thrB*). This strain was derived from the known strain C600 (*thrB, leuB*) (Appleyard R.K., Genetics, 39, 440-452 (1954)) as Leu^+ revertant. A transformant strain of 44 being transformed with a plasmid containing a *thrA* gene coding for aspartokinase-homoserine dehydrogenase I may be preferably used.

[0037] A strain of Escherichia coli 44 has been deposited in Russian National Collection of Industrial Microorganisms (VKPM) Depositary, GNIIgenetika; 1, Dorozhny Proezd., 1, 113545, Moscow, Russia, since September 23, 1980 under a Registration number of VKPM B-2175, and transferred from the original deposit to international deposit based on Budapest Treaty on September 1, 2000.

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(4) L-glutamic acid-producing bacteria

[0038] The L-glutamic acid producing bacteria belonging to the genus *Escherichia* may be exemplified by a strain which is resistant to L-valine, for example, *Escherichia coli* strains B11, K-12 (ATCC10798), B (ATCC11303) and W (ATCC9637).

(5) L-isoleucine-producing bacteria

[0039] The L-isoleucine-producing bacteria belonging to the genus *Escherichia* may be exemplified by a strain TDH6/pVIC40, pMWD5 (Hashiguchi, K. et al., Biosci. Biotechnol. Biochem., 63(4), 672-679 (1999)) or AJ12919 described in EP0 685 555 A1.

(2) Method for producing an L-amino acid

[0040] An L-amino acid can be efficiently produced by cultivating a bacterium belonging to the genus *Escherichia* which is transformed with *pycA* gene as described above and has an L-amino acid productivity in an appropriate medium, producing and accumulating the L-amino acid in the medium, and collecting the L-amino acid from the medium.

[0041] The L-amino acid is exemplified preferably by L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine. The L-amino acid may be produced by the method of the present invention either alone or as a mixture of more than two kinds of L-amino acids.

[0042] In the method of present invention, the cultivation of the bacterium belonging to the genus *Escherichia*, the collection and purification of amino acid from the liquid medium may be performed in a manner similar to those of the conventional method for producing an amino acid by fermentation using a bacterium. A medium used in cultivation may be either a synthetic medium or a natural medium, so long as the medium includes a carbon and a nitrogen source and minerals and, if necessary, nutrients which the bacterium used requires for growth in appropriate amounts. The carbon source may include various carbohydrates such as glucose and sucrose, and various organic acids. Depending on assimilatory ability of the used bacterium, alcohol including ethanol and glycerol may be used. As the nitrogen source, ammonia, various ammonium salts such as ammonium sulfate, other nitrogen compounds such as amines, a natural nitrogen source such as peptone, soybean hydrolyzate and digested fermentative microbe are used. As minerals, monopotassium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, calcium carbonate are used.

[0043] The cultivation is preferably performed under an aerobic condition such as a shaking culture, and an aeration and stirring culture. The temperature of culture is usually 20 to 40°C, preferably 30 to 38°C. The pH of the culture is usually between 5 and 9, preferably between 6.5 and 7.2. The pH of the culture can be adjusted with ammonia, calcium carbonate, various acids, various bases, and buffers. Usually, 1 to 3-day cultivation leads to the accumulation of the target L-amino acid in the medium.

[0044] Collecting and purifiying the L-amino acid can be performed by removing solids such as cells from the medium by centrifugation or membrane filtration after cultivation, followed by ion exchange, concentration and crystalline fraction methods and the like.

Best Mode for Carrying Out the Invention

[0045] The present invention will be more concretely explained below with reference to Examples.

Example 1: Cloning of the pycA gene from Bacillus subtilis 168 strain

(1) Construction of *B. subtilis* pycA::KmR recipient strain for cloning

[0046] The recipient strain of *B. subtilis* was constructed by insertional mutagenesis of pycA gene. The fragment of pycA gene was cloned in two steps (refer to Figs. 1 and 2).

[0047] In a data bank one of reading frame (ylaP) from *B. subtilis* genome sequence project is identified as *pycA* gene, encoding pyruvate carboxylase . The region located immediately after the *pycA* gene terminator was cloned by PCR amplification, using the following pair of oligonucleotides:

5' -GATATAAGGGGACTTCAGAG, and
5' -GGCGCTTTATGCGTTTCAATC.

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[0048] The 269bp DNA fragment was blunt-ended with Klenow fragment of DNA polymerase I and cloned into *Sca*I site of pBR322 plasmid in *E. coli* strain. The resulting plasmid pBRPYCA11 was digested by *Cla*I and ligated to 1628bp DNA fragment carrying *cat*-gene, which was obtained by digestion of pC194 with *Cla*I. Then, using the obtained plasmid pBRPYCA11CmR3, *Bacillus subtilis* 168 trpC2 strain was transformed and Cm^R -clones were selected. The plasmid pBRPYCA11CmR3 cannot autonomously replicate in baterium belonging to the genus *Bacillus*, but can integrate into chromosome near the *pycA* gene by homology with the 269bp DNA fragment. The strain was named as *B. subtilis* trpC2 xyz::pBRPYCACmR3. The integrant carried pBR322-replicon, *tet* gene encoding resistance to tetracycline derived from pBR322 and Cm^R marker.

[0049] Chromosomal DNA of *B. subtilis* trpC2 xyz::pBRPYCACmR3 was isolated and used to clone a distal part of *pycA* gene. The chromosomal DNA which is digested with *Pst*I was self-ligated and transformed into *E. coli* TG1 strain. Recombinants resitant to tretracycline and chloramphenicol were selected. The plasmid was recovered from one of the recombinant and named as pPYC1. The plasmid pPYC1 contained a single *Bgl*II restriction site, which located in *pycA* gene fragment. This site was used to insert 1818bp DNA fragment, containing a kanamycine resistance marker for *B. subtilis*. The plasmid pPYC1 was digested with *Bgl*II and ligated to pUC7KmR which was digested with *Bam*HI to obtain a plasmid pPYC1::KmR.

[0050] The inactivation of *pycA* gene in a wild type *B. subtilis* 168 trpC2 was achieved by transformation of the same strain with linerized plasmid pPYC1::Km^R by one step procedure, followed by selection on Luria broth supplemented with kanamycin. The Km^R marker was inserted into pycA gene on chromosomal DNA by homologous double-crossover recombination.

[0051] The constructed strain of *B. subtilis* pycA::Km^R trpC2 did not grow in Spizizen minimal medium (Anagnostopoulos, C and Spizizen, J., J. Bacteriol., 81, 741-746 (1961)) with tryptophan unless citrate or aspartate were added to the media.

(Composition of Spizizen minimal medium)

[0052]

$K_2HPO_4 \cdot 3H_2O$	18.3 g/L
KH_2PO_4	6.0 g/L
$(NH4)_2SO_4$	2.0 g/L
sodium citrate $\cdot 5H_2O$	1.2 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
Glucose	10.0 g/L
pH7.0	

[0053] The *B. subtilis* trpC2 pycA::Km^R recE::Tc^R strain was constructed by transducing the inactivated allele of *recE* gene from *B. subtilis* recE::Tc^R strain with phage E40 (Fig. 2). It was necessary to inactivate recombination in recipient strain for cloning of *pycA* gene from *B. subtilis*. The constructed strain *B. subtilis* trpC2 pycA::Km^R recE::Tc^R strain was used as recipient to clone pycA gene.

(2) Cloning of *B. subtilis* native *pycA* gene by complementation.

[0054] The *pycA* gene was cloned from *B. subtilis* 168 trpC2 strain. Chromosomal DNA prepared from the strain 168 trpC2 was partially digested with *Eco*RI and ligated to pCB20 (EmR) (gifted by Dr. Yu Jomantas, refer to "Genetic transformation and expression " L.O. Butler, et al. eds., Intercept Ltd., PO Box 402, Wimborne, Dorset, BH229T2, U.K., 1989, p269-281) which was digested with *Eco*RI. Then the trpC2 pycA::Km^R recE::Tc^R recipient strain was transformed with the ligation mixture (Fig. 2). The Asp^+,Em^R,Km^R clones were selected on Spizizen minimal medium without citrate or aspartate supplementation but suplemented with tryptophan, erythromycin and kanamycin. The plasmid pPYCR3 harbored by one of the selected clones comlemented *pycA* mutation, had a predicted structure and conferred the same Asp^+, Em^R phenotype after second transformation.

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(3) Cloning of DNA fragment carrying *pycA* gene from *B.* subtilis 168 trpC2 strain into high copy number plasmid pUC18 and pUC19

[0055] Plasmid pPYCR3 was digested with *Hind*II and *Sca*I and *Hind*II-*Sca*I fragment (4414 bp), carrying *pycA* gene with 5'-end upstream sequence (including promoter and ribosome binding site), was cloned in pUC18 digested with *Sma*I. Thus the plasmid pUC18-pycA was obatined (Fig. 3, upside). The ligation reaction was performed concretely as follows. Ninety ng of *Sma*I-digested pUC18 and 300 ng of *Hind*II-*Sca*I fragment (4414 bp) carrying pycA gene which was purified by agarose gel electrophresis were ligated with 2 units of T4 DNA Ligase (Pharmacia, Sweden) in 45 µl of reaction mixture containing 1x One-Phor-All buffer (Pharmacia, Sweden) and 1 mM ATP.

[0056] *E. coli* TG1 was transformed with pUC18-pycA. Obtained transformants were analyzed by using *Kpn*I, *Xba*I, *Bam*HI, *Hind*III and *Eco*RI endonucleases. All clones carried the *pycA* gene in opposite direction to *lac* promoter of pUC18 plasmid. However, the *pycA* genes cloned in pUC18 may be expressed under the control of the inherent promoter of the gene.

[0057] Then in order to rearrange *pycA* gene under control of *lac* promoter, the *Kpn*I-*Xba*I fragment of pUC18-pycA was cloned into pUC19 digested with *Kpn*I-*Xba*I. However, the obtained clones were unstable and the recombinant plasmid was eliminated during short time (6-8 h) of cultivation.

(4) Cloning of DNA fragment carrying *pycA* gene into a low copy number plasmid pMW119

[0058] The *Kpn*I-*Xba*I fragment of pUC18-pycA was ligated to a low copy number plasmid pMW119 digested with *Kpn*I and *Xba*I (Fig. 3, downside). The ligation reaction was performed in 50 µl of reaction mixture containing 60 ng of pMW119 digested with *Kpn*I-*Xba*I, 120 ng of *Kpn*I-*Xba*I fragment of pUC18-pycA which was purified by agarose gel electrophresis, 1x One-Phor-All buffer (Pharmacia, Sweden), 1 mM ATP and 1 unit of T4 DNA Ligase (Pharmacia, Sweden).

[0059] Using the ligation mixture, *E. coli* TG1 was transformed. Resulting transformants were analyzed using *Sac*I, *Kpn*I, *Xba*I, *Bam*HI, *Hind*III and *Eco*RI. All clones carried *pycA* gene under control of *lac* promoter of pMW119 and its own promoter. The thus obtained clone was named TG1(pMW119-pycA), from which the plasmid pMW119-pycA was obatined.

Example 2: Production of L-threonine and L-glutamic acid

[0060] E. coli strain MG442 was transformed with the plasmid pMW119-pycA and 10 Amp^r colonies were sellected. Productivities of L-threonine and L-glutamic acid of the colonies were examined using the following fermentation midium.

(Composition of fermentation medium)

[0061]

glucose (separately sterilized)	60 g/L
$(NH4)_2SO_4$	15.0 g/L
KH_2PO_4	1.5 g/L
$MgSO_4 \cdot 7H_2O$	1.0 g/L
L-isoleucine	100 mg/L
thiamine	0.1 mg/L
$CaCO_3$ (separately sterilized)	20 g/L

[0062] Fermentation was performed in test tubes (20x300mm) at 32 °C for 72 hours using a rotary shaker. One loop of each of 10 clones was inoculated into the test tube containing 2.0 ml of the fermentation medium. As controls, 10 single colonies of a plasmid-less strain MG442 were used. After the fermention, concentrations of L-threonine and L-glutamic acid accumulated in the media were mesured by thin-layer chromatography. The results are shown in Table 1.

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Table 1

Clone Nos.	MG442(control)		MG442(pMW119-pycA)	
	L-thr G/L	L-glu G/L	L-thr g/L	L-glu g/L
1	5.5	9.5	7.8	12.3
2	3.2	7.1	9.8	12.3
3	3.6	7.1	9.4	13.3
4	3.4	8.9	9.8	11.8
5	3.3	8.9	9.8	11.8
6	4.8	7.3	9.9	11.8
7	3.3	7.0	10.0	10.7
8	4.7	8.3	10.0	10.4
9	3.2	7.3	9.7	11.2
10	2.6	4.1	10.0	10.7
Average	3.8	7.4	9.6	11.6

Example 3: Production of L-homoserine

[0063] L-homoserine-producing E. coli strain 44 was transformed with the plasmid pMW119-pycA and five ampiciline resistant clones were selected. Production of L-homoserine was examined as it is indicated in the Example 1, but the fermentation medium was supplemented with 0.5 g/l of L-threonine. As a control five colonies of plasmidless strain 44 were used. After the fermentation average L-homoserine concentrations were 4.5 g/l (the strain 44) and 5.7 g/l (the starin 44 with the plasmid pMW119-pycA).

[0064] Also the effect of pycA gene on the productivity of L-lysine is reasonably expected, because pycA gene is confirmed to have the positive effect on the productivity of L-threonine and L-homoserine which share the same biosynthetic pathway in part as L-lysine.

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SEQUENCE LISTING

<110> Ajinomoto Co., Inc.

<120> Method for Producing L- Amino Acid by Fermentation

<130> EPA-53466

<150> RU 99121636
<151> 1999-10-14

<160> 4

<170> PatentIn Ver. 2.0

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<212> DNA
<213> Artificial Sequence

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<210> 2
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Asn Arg Gly Glu Ile Ala Ile Arg Ile Phe Arg Ala Cys Thr Glu Leu
15 20 25

aat att cgt aca gtt gcg gtc tat tca aaa gaa gat tcc ggt tcc tac 147
Asn Ile Arg Thr Val Ala Val Tyr Ser Lys Glu Asp Ser Gly Ser Tyr
30 35 40

cat cgg tac aaa gcg gat gaa gca tac ttg gtc ggt gaa ggg aaa aaa 195
His Arg Tyr Lys Ala Asp Glu Ala Tyr Leu Val Gly Glu Gly Lys Lys
45 50 55 60

ccg att gat gct tac ctg gat att gaa ggt atc att gat att gcg aaa 243
Pro Ile Asp Ala Tyr Leu Asp Ile Glu Gly Ile Ile Asp Ile Ala Lys
65 70 75

aga aac aaa gtc gat gca att cat ccg gga tac ggt ttc tta tct gaa 291
Arg Asn Lys Val Asp Ala Ile His Pro Gly Tyr Gly Phe Leu Ser Glu
80 85 90

aat att cat ttt gcg aga cga tgt gaa gaa gaa ggc atc gta ttc ata 339

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ggg cca aaa tcc gag cat ctc gat atg ttt ggt gac aag gta aaa gcg   387
Gly Pro Lys Ser Glu His Leu Asp Met Phe Gly Asp Lys Val Lys Ala
    110                 115                 120
cgt gag cag gca gaa aaa gcg gga atc ccc gtg att ccg gga agc gac   435
Arg Glu Gln Ala Glu Lys Ala Gly Ile Pro Val Ile Pro Gly Ser Asp
125                 130                 135                 140
ggt cct gcc gaa acg ctt gaa gcc gtc gaa caa ttt gga caa gct aac   483
Gly Pro Ala Glu Thr Leu Glu Ala Val Glu Gln Phe Gly Gln Ala Asn
                145                 150                 155
ggt tat ccg atc atc att aaa gcc tcg ctt ggc ggc ggc ggc cgc ggt   531
Gly Tyr Pro Ile Ile Ile Lys Ala Ser Leu Gly Gly Gly Gly Arg Gly
            160                 165                 170
atg cgg att gtc aga tct gaa agt gaa gtt aaa gaa gca tat gag cgt   579
Met Arg Ile Val Arg Ser Glu Ser Glu Val Lys Glu Ala Tyr Glu Arg
        175                 180                 185
gct aaa tca gag gcg aaa gca gcc ttt ggc aat gat gaa gtt tat gta   627
Ala Lys Ser Glu Ala Lys Ala Ala Phe Gly Asn Asp Glu Val Tyr Val
    190                 195                 200
gaa aaa tta att gag aat ccg aaa cat att gag gtt cag gtc att gga   675
Glu Lys Leu Ile Glu Asn Pro Lys His Ile Glu Val Gln Val Ile Gly
205                 210                 215                 220
gac aag cag ggc aat gtc gtc cat ctt ttt gag agg gat tgc tcc gtt   723
Asp Lys Gln Gly Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Val
                225                 230                 235
caa aga cgc cat caa aaa gtc att gaa gtg gcg ccg agt gtc tcg ctg   771
Gln Arg Arg His Gln Lys Val Ile Glu Val Ala Pro Ser Val Ser Leu
            240                 245                 250
tca cct gaa tta agg gac caa att tgt gag gct gca gtt gcg ctt gcc   819
Ser Pro Glu Leu Arg Asp Gln Ile Cys Glu Ala Ala Val Ala Leu Ala
        255                 260                 265
aaa aat gta aac tat ata aat gcg ggg acg gtc gaa ttc ctt gtt gca   867
Lys Asn Val Asn Tyr Ile Asn Ala Gly Thr Val Glu Phe Leu Val Ala
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    270                 275                 280
aac aac gag ttc tac ttt att gaa gta aat cct cgc gta caa gtt gaa   915
Asn Asn Glu Phe Tyr Phe Ile Glu Val Asn Pro Arg Val Gln Val Glu
285                 290                 295                 300
cac acg ata aca gaa atg att act ggt gtc gat att gtt caa act cag   963
His Thr Ile Thr Glu Met Ile Thr Gly Val Asp Ile Val Gln Thr Gln
                305                 310                 315
atc ctt gtt gcc caa ggg cac agc ctt cac agc aaa aaa gta aat att   1011
Ile Leu Val Ala Gln Gly His Ser Leu His Ser Lys Lys Val Asn Ile
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cct gag caa aag gac att ttt aca atc ggc tat gcc att cag tca cgg   1059
Pro Glu Gln Lys Asp Ile Phe Thr Ile Gly Tyr Ala Ile Gln Ser Arg
        335                 340                 345
gtt acg act gag gat ccg caa aat gat ttc atg cct gat aca gga aaa   1107
Val Thr Thr Glu Asp Pro Gln Asn Asp Phe Met Pro Asp Thr Gly Lys
    350                 355                 360
atc atg gct tac cgc tca ggc ggc ggt ttt ggt gtc cgt ctt gat acc   1155
Ile Met Ala Tyr Arg Ser Gly Gly Gly Phe Gly Val Arg Leu Asp Thr
365                 370                 375                 380
gga aac agc ttc cag ggc gcc gtg atc aca cca tac tat gat tca ctt   1203
Gly Asn Ser Phe Gln Gly Ala Val Ile Thr Pro Tyr Tyr Asp Ser Leu
                385                 390                 395
ctc gtt aag ctt tca act tgg gct tta acg ttt gaa cag gca gct gcc   1251
Leu Val Lys Leu Ser Thr Trp Ala Leu Thr Phe Glu Gln Ala Ala Ala
            400                 405                 410
aaa atg gtg cga aac ctt cag gag ttt aga atc aga ggc ata aaa acg   1299
Lys Met Val Arg Asn Leu Gln Glu Phe Arg Ile Arg Gly Ile Lys Thr
        415                 420                 425
aac att ccg ttc ctt gag aac gtt gca aag cat gag aag ttc ctg aca   1347
Asn Ile Pro Phe Leu Glu Asn Val Ala Lys His Glu Lys Phe Leu Thr
    430                 435                 440
ggg caa tat gat aca tct ttc att gat aca acg cct gaa tta ttt aat   1395
Gly Gln Tyr Asp Thr Ser Phe Ile Asp Thr Thr Pro Glu Leu Phe Asn
445                 450                 455                 460
```


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Phe Pro Lys Gln Lys Asp Arg Gly Thr Lys Met Leu Thr Tyr Ile Gly
                465                 470                 475

aat gtg aca gtg aac ggc ttc cct gga atc ggg aaa aaa gaa aaa ccg   1491
Asn Val Thr Val Asn Gly Phe Pro Gly Ile Gly Lys Lys Glu Lys Pro
            480                 485                 490

gcg ttt gac aaa ccg tta ggc gta aag gta gac gtt gat cag cag cct   1539
Ala Phe Asp Lys Pro Leu Gly Val Lys Val Asp Val Asp Gln Gln Pro
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gcc aga gga aca aag caa att ctc gat gaa aaa ggt gca gaa ggg ctt   1587
Ala Arg Gly Thr Lys Gln Ile Leu Asp Glu Lys Gly Ala Glu Gly Leu
    510                 515                 520

gca aat tgg gtt aag gag cag aaa tct gtc ctt tta act gat acg aca   1635
Ala Asn Trp Val Lys Glu Gln Lys Ser Val Leu Leu Thr Asp Thr Thr
525                 530                 535                 540

ttc agg gat gcc cac caa tcg tta ttg gca act aga atc aga tcg cat   1683
Phe Arg Asp Ala His Gln Ser Leu Leu Ala Thr Arg Ile Arg Ser His
                545                 550                 555

gat ttg aaa aaa atc gca aat ccg acg gct gcg tta tgg cct gaa cta   1731
Asp Leu Lys Lys Ile Ala Asn Pro Thr Ala Ala Leu Trp Pro Glu Leu
            560                 565                 570

ttc agt atg gaa atg tgg gga ggc gcg acc ttc gat gta gcc tac cga   1779
Phe Ser Met Glu Met Trp Gly Gly Ala Thr Phe Asp Val Ala Tyr Arg
        575                 580                 585

ttc ctg aaa gaa gat ccg tgg aaa cgt ttg gaa gat ctt cgc aaa gaa   1827
Phe Leu Lys Glu Asp Pro Trp Lys Arg Leu Glu Asp Leu Arg Lys Glu
    590                 595                 600

gtg ccg aat acc tta ttc cag atg ttg ctt cgc tca tca aat gcg gtc   1875
Val Pro Asn Thr Leu Phe Gln Met Leu Leu Arg Ser Ser Asn Ala Val
605                 610                 615                 620

ggc tat acg aat tat ccg gac aat gtg att aaa gaa ttt gtg aag caa   1923
Gly Tyr Thr Asn Tyr Pro Asp Asn Val Ile Lys Glu Phe Val Lys Gln
                625                 630                 635

tca gct caa tcc ggt att gat gtg ttt cgt att ttc gac agc tta aac   1971
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Ser Ala Gln Ser Gly Ile Asp Val Phe Arg Ile Phe Asp Ser Leu Asn
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tgg gta aaa ggg atg acg tta gcc att gat gct gtt agg gat acc ggc   2019
Trp Val Lys Gly Met Thr Leu Ala Ile Asp Ala Val Arg Asp Thr Gly
       655                 660                 665
aaa gtg gca gaa gct gcg att tgt tat acg gga gat atc ctt gac aag   2067
Lys Val Ala Glu Ala Ala Ile Cys Tyr Thr Gly Asp Ile Leu Asp Lys
   670                 675                 680
aac cgg acg aag tac gac ctt gca tat tat aca tcg atg gcg aag gag   2115
Asn Arg Thr Lys Tyr Asp Leu Ala Tyr Tyr Thr Ser Met Ala Lys Glu
685                 690                 695                 700
ctt gag gcg gcc gga gcc cat att ctc ggg att aaa gat atg gca ggg   2163
Leu Glu Ala Ala Gly Ala His Ile Leu Gly Ile Lys Asp Met Ala Gly
               705                 710                 715
ctg tta aaa ccg cag gct gca tat gag ctc gtt tct gcg ttg aaa gaa   2211
Leu Leu Lys Pro Gln Ala Ala Tyr Glu Leu Val Ser Ala Leu Lys Glu
          720                 725                 730
acg atc gac att ccg gtt cac ctt cat acg cat gat acg agc gga aac   2259
Thr Ile Asp Ile Pro Val His Leu His Thr His Asp Thr Ser Gly Asn
       735                 740                 745
ggt att tat atg tat gcg aaa gct gtt gaa gcc ggc gtt gat atc ata   2307
Gly Ile Tyr Met Tyr Ala Lys Ala Val Glu Ala Gly Val Asp Ile Ile
   750                 755                 760
gac gtg gcg gtc agc tca atg gcg gga tta acg tca cag cct agc gcg   2355
Asp Val Ala Val Ser Ser Met Ala Gly Leu Thr Ser Gln Pro Ser Ala
765                 770                 775                 780
agc gga ttt tat cat gcg atg gaa ggc aac gac cgc cgt ccg gaa atg   2403
Ser Gly Phe Tyr His Ala Met Glu Gly Asn Asp Arg Arg Pro Glu Met
               785                 790                 795
aat gtc caa ggc gtt gaa ttg ctg tcc caa tat tgg gag tcg gtg cgt   2451
Asn Val Gln Gly Val Glu Leu Leu Ser Gln Tyr Trp Glu Ser Val Arg
          800                 805                 810
aaa tat tat agt gaa ttt gaa agc gga atg aag tct ccg cat act gaa   2499
Lys Tyr Tyr Ser Glu Phe Glu Ser Gly Met Lys Ser Pro His Thr Glu
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Ile Tyr Glu His Glu Met Pro Gly Gly Gln Tyr Ser Asn Leu Gln Gln
    830                 835                 840
caa gcc aag gga gta ggc ctt ggc gac cgc tgg aac gaa gtc aag gaa   2595
Gln Ala Lys Gly Val Gly Leu Gly Asp Arg Trp Asn Glu Val Lys Glu
845                 850                 855                 860
atg tac aga cgc gtg aac gat atg ttc ggt gac atc gtc aag gta acg   2643
Met Tyr Arg Arg Val Asn Asp Met Phe Gly Asp Ile Val Lys Val Thr
                865                 870                 875
cct tcc tca aaa gta gtc gga gat atg gca ctc tac atg gtg caa aac   2691
Pro Ser Ser Lys Val Val Gly Asp Met Ala Leu Tyr Met Val Gln Asn
            880                 885                 890
aat ctg act gaa aaa gac gtt tac gaa aaa ggt gaa tct tta gat ttc   2739
Asn Leu Thr Glu Lys Asp Val Tyr Glu Lys Gly Glu Ser Leu Asp Phe
        895                 900                 905
cct gat tct gtc gtg gag ctt ttt aaa gga aat atc ggc cag cct cat   2787
Pro Asp Ser Val Val Glu Leu Phe Lys Gly Asn Ile Gly Gln Pro His
    910                 915                 920
ggc gga ttc cca gaa aaa ctg caa aag ctg atc tta aaa ggg cag gag   2835
Gly Gly Phe Pro Glu Lys Leu Gln Lys Leu Ile Leu Lys Gly Gln Glu
925                 930                 935                 940
ccg att aca gtc aga ccg ggc gaa ctg ctt gag ccg gtg tca ttt gaa   2883
Pro Ile Thr Val Arg Pro Gly Glu Leu Leu Glu Pro Val Ser Phe Glu
                945                 950                 955
gcg atc aaa cag gaa ttt aaa gag cag cat aac ttg gaa att tca gat   2931
Ala Ile Lys Gln Glu Phe Lys Glu Gln His Asn Leu Glu Ile Ser Asp
            960                 965                 970
cag gat gct gtg gca tat gcc ctt tat cct aaa gtc ttc act gat tat   2979
Gln Asp Ala Val Ala Tyr Ala Leu Tyr Pro Lys Val Phe Thr Asp Tyr
        975                 980                 985
gtg aaa acg aca gaa agc tat gga gac atc tcg gta tta gat aca ccg   3027
Val Lys Thr Thr Glu Ser Tyr Gly Asp Ile Ser Val Leu Asp Thr Pro
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1005                1010                1015                1020
gag cgc ggc aaa acg ctg atc gtt aag ctg att tca atc ggt gag cct   3123
Glu Arg Gly Lys Thr Leu Ile Val Lys Leu Ile Ser Ile Gly Glu Pro
                1025                1030                1035
cag cct gat gcc acc cgc gtc gtt tat ttc gaa ctc aac ggg cag ccg   3171
Gln Pro Asp Ala Thr Arg Val Val Tyr Phe Glu Leu Asn Gly Gln Pro
            1040                1045                1050
cgt gaa gta gtc att aaa gat gaa agc att aag tct tcc gtt cag gaa   3219
Arg Glu Val Val Ile Lys Asp Glu Ser Ile Lys Ser Ser Val Gln Glu
        1055                1060                1065
agg ctg aaa gca gac cgg aca aat cca agc cac atc gca gct tcc atg   3267
Arg Leu Lys Ala Asp Arg Thr Asn Pro Ser His Ile Ala Ala Ser Met
    1070                1075                1080
cct gga aca gtt att aag gta ttg gct gaa gca ggc aca aaa gtc aat   3315
Pro Gly Thr Val Ile Lys Val Leu Ala Glu Ala Gly Thr Lys Val Asn
1085                1090                1095                1100
aaa ggt gat cat ttg atg att aat gaa gcg atg aaa atg gaa aca acg   3363
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Val Gln Ala Pro Phe Ser Gly Thr Ile Lys Gln Val His Val Lys Asn
            1120                1125                1130
ggt gag ccg atc caa acg gga gat ctg ctc ctt gaa att gaa aaa gca   3459
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taa                                                              3462
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<210> 4
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Val Ala Val Tyr Ser Lys Glu Asp Ser Gly Ser Tyr His Arg Tyr Lys
35 40 45
Ala Asp Glu Ala Tyr Leu Val Gly Glu Gly Lys Lys Pro Ile Asp Ala
50 55 60
Tyr Leu Asp Ile Glu Gly Ile Ile Asp Ile Ala Lys Arg Asn Lys Val
65 70 75 80
Asp Ala Ile His Pro Gly Tyr Gly Phe Leu Ser Glu Asn Ile His Phe
85 90 95
Ala Arg Arg Cys Glu Glu Glu Gly Ile Val Phe Ile Gly Pro Lys Ser
100 105 110
Glu His Leu Asp Met Phe Gly Asp Lys Val Lys Ala Arg Glu Gln Ala
115 120 125
Glu Lys Ala Gly Ile Pro Val Ile Pro Gly Ser Asp Gly Pro Ala Glu
130 135 140
Thr Leu Glu Ala Val Glu Gln Phe Gly Gln Ala Asn Gly Tyr Pro Ile
145 150 155 160
Ile Ile Lys Ala Ser Leu Gly Gly Gly Gly Arg Gly Met Arg Ile Val
165 170 175
Arg Ser Glu Ser Glu Val Lys Glu Ala Tyr Glu Arg Ala Lys Ser Glu
180 185 190
Ala Lys Ala Ala Phe Gly Asn Asp Glu Val Tyr Val Glu Lys Leu Ile
195 200 205
Glu Asn Pro Lys His Ile Glu Val Gln Val Ile Gly Asp Lys Gln Gly
210 215 220
Asn Val Val His Leu Phe Glu Arg Asp Cys Ser Val Gln Arg Arg His
225 230 235 240
Gln Lys Val Ile Glu Val Ala Pro Ser Val Ser Leu Ser Pro Glu Leu
245 250 255
Arg Asp Gln Ile Cys Glu Ala Ala Val Ala Leu Ala Lys Asn Val Asn

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260 265 270

Tyr Ile Asn Ala Gly Thr Val Glu Phe Leu Val Ala Asn Asn Glu Phe

275 280 285

Tyr Phe Ile Glu Val Asn Pro Arg Val Gln Val Glu His Thr Ile Thr

290 295 300

Glu Met Ile Thr Gly Val Asp Ile Val Gln Thr Gln Ile Leu Val Ala

305 310 315 320

Gln Gly His Ser Leu His Ser Lys Lys Val Asn Ile Pro Glu Gln Lys

325 330 335

Asp Ile Phe Thr Ile Gly Tyr Ala Ile Gln Ser Arg Val Thr Thr Glu

340 345 350

Asp Pro Gln Asn Asp Phe Met Pro Asp Thr Gly Lys Ile Met Ala Tyr

355 360 365

Arg Ser Gly Gly Gly Phe Gly Val Arg Leu Asp Thr Gly Asn Ser Phe

370 375 380

Gln Gly Ala Val Ile Thr Pro Tyr Tyr Asp Ser Leu Leu Val Lys Leu

385 390 395 400

Ser Thr Trp Ala Leu Thr Phe Glu Gln Ala Ala Ala Lys Met Val Arg

405 410 415

Asn Leu Gln Glu Phe Arg Ile Arg Gly Ile Lys Thr Asn Ile Pro Phe

420 425 430

Leu Glu Asn Val Ala Lys His Glu Lys Phe Leu Thr Gly Gln Tyr Asp

435 440 445

Thr Ser Phe Ile Asp Thr Thr Pro Glu Leu Phe Asn Phe Pro Lys Gln

450 455 460

Lys Asp Arg Gly Thr Lys Met Leu Thr Tyr Ile Gly Asn Val Thr Val

465 470 475 480

Asn Gly Phe Pro Gly Ile Gly Lys Lys Glu Lys Pro Ala Phe Asp Lys

485 490 495

Pro Leu Gly Val Lys Val Asp Val Asp Gln Gln Pro Ala Arg Gly Thr

500 505 510

Lys Gln Ile Leu Asp Glu Lys Gly Ala Glu Gly Leu Ala Asn Trp Val

515 520 525

Lys Glu Gln Lys Ser Val Leu Leu Thr Asp Thr Thr Phe Arg Asp Ala

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His Gln Ser Leu Leu Ala Thr Arg Ile Arg Ser His Asp Leu Lys Lys
545                 550                 555                 560
Ile Ala Asn Pro Thr Ala Ala Leu Trp Pro Glu Leu Phe Ser Met Glu
                565                 570                 575
Met Trp Gly Gly Ala Thr Phe Asp Val Ala Tyr Arg Phe Leu Lys Glu
            580                 585                 590
Asp Pro Trp Lys Arg Leu Glu Asp Leu Arg Lys Glu Val Pro Asn Thr
        595                 600                 605
Leu Phe Gln Met Leu Leu Arg Ser Ser Asn Ala Val Gly Tyr Thr Asn
    610                 615                 620
Tyr Pro Asp Asn Val Ile Lys Glu Phe Val Lys Gln Ser Ala Gln Ser
625                 630                 635                 640
Gly Ile Asp Val Phe Arg Ile Phe Asp Ser Leu Asn Trp Val Lys Gly
                645                 650                 655
Met Thr Leu Ala Ile Asp Ala Val Arg Asp Thr Gly Lys Val Ala Glu
            660                 665                 670
Ala Ala Ile Cys Tyr Thr Gly Asp Ile Leu Asp Lys Asn Arg Thr Lys
        675                 680                 685
Tyr Asp Leu Ala Tyr Tyr Thr Ser Met Ala Lys Glu Leu Glu Ala Ala
    690                 695                 700
Gly Ala His Ile Leu Gly Ile Lys Asp Met Ala Gly Leu Leu Lys Pro
705                 710                 715                 720
Gln Ala Ala Tyr Glu Leu Val Ser Ala Leu Lys Glu Thr Ile Asp Ile
                725                 730                 735
Pro Val His Leu His Thr His Asp Thr Ser Gly Asn Gly Ile Tyr Met
            740                 745                 750
Tyr Ala Lys Ala Val Glu Ala Gly Val Asp Ile Ile Asp Val Ala Val
        755                 760                 765
Ser Ser Met Ala Gly Leu Thr Ser Gln Pro Ser Ala Ser Gly Phe Tyr
    770                 775                 780
His Ala Met Glu Gly Asn Asp Arg Arg Pro Glu Met Asn Val Gln Gly
785                 790                 795                 800
Val Glu Leu Leu Ser Gln Tyr Trp Glu Ser Val Arg Lys Tyr Tyr Ser
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805 810 815

Glu Phe Glu Ser Gly Met Lys Ser Pro His Thr Glu Ile Tyr Glu His

820 825 830

Glu Met Pro Gly Gly Gln Tyr Ser Asn Leu Gln Gln Gln Ala Lys Gly

835 840 845

Val Gly Leu Gly Asp Arg Trp Asn Glu Val Lys Glu Met Tyr Arg Arg

850 855 860

Val Asn Asp Met Phe Gly Asp Ile Val Lys Val Thr Pro Ser Ser Lys

865 870 875 880

Val Val Gly Asp Met Ala Leu Tyr Met Val Gln Asn Asn Leu Thr Glu

885 890 895

Lys Asp Val Tyr Glu Lys Gly Glu Ser Leu Asp Phe Pro Asp Ser Val

900 905 910

Val Glu Leu Phe Lys Gly Asn Ile Gly Gln Pro His Gly Gly Phe Pro

915 920 925

Glu Lys Leu Gln Lys Leu Ile Leu Lys Gly Gln Glu Pro Ile Thr Val

930 935 940

Arg Pro Gly Glu Leu Leu Glu Pro Val Ser Phe Glu Ala Ile Lys Gln

945 950 955 960

Glu Phe Lys Glu Gln His Asn Leu Glu Ile Ser Asp Gln Asp Ala Val

965 970 975

Ala Tyr Ala Leu Tyr Pro Lys Val Phe Thr Asp Tyr Val Lys Thr Thr

980 985 990

Glu Ser Tyr Gly Asp Ile Ser Val Leu Asp Thr Pro Thr Phe Phe Tyr

995 1000 1005

Gly Met Thr Leu Gly Glu Glu Ile Glu Val Glu Ile Glu Arg Gly Lys

1010 1015 1020

Thr Leu Ile Val Lys Leu Ile Ser Ile Gly Glu Pro Gln Pro Asp Ala

025 1030 1035 1040

Thr Arg Val Val Tyr Phe Glu Leu Asn Gly Gln Pro Arg Glu Val Val

1045 1050 1055

Ile Lys Asp Glu Ser Ile Lys Ser Ser Val Gln Glu Arg Leu Lys Ala

1060 1065 1070

Asp Arg Thr Asn Pro Ser His Ile Ala Ala Ser Met Pro Gly Thr Val

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1075 1080 1085

Ile Lys Val Leu Ala Glu Ala Gly Thr Lys Val Asn Lys Gly Asp His

1090 1095 1100

Leu Met Ile Asn Glu Ala Met Lys Met Glu Thr Thr Val Gln Ala Pro

105 1110 1115 1120

Phe Ser Gly Thr Ile Lys Gln Val His Val Lys Asn Gly Glu Pro Ile

1125 1130 1135

Gln Thr Gly Asp Leu Leu Leu Glu Ile Glu Lys Ala

1140 1145

Claims

1. A bacterium belonging to the genus Escherichia which contains a gene coding for an enzyme having pyruvate carboxylase activity and which has L-amino acid productivity.

2. The bacterium according to claim 1, wherein the gene coding for an enzyme having pyruvate carboxylase activity is pyruvate carboxylase derived from a bacterium belonging to the genus Bacillus.

3. The bacterium according to claim 1 or 2, wherein said gene has the nucleotide sequence as shown in SEQ ID NO 3.

4. The bacterium accodring to any of the preceding claims, wherein said enzyme has an amino acid sequence as shown in SEQ ID NO 4.

5. The bacterium according to any of the preceding claims which is transformed with a vector containing said gene.

6. The bacterium according to any of the preceding claims, wherein the gene is introduced into said bacterium in a low copy number.

7. The bacterium according to any of the preceding claims, wherein the L-amino acid is selected from the group consisting of L-threonine, L-lysine, L-glutamic acid, L-homoserine, L-methionine, L-arginine, L-proline and L-isoleucine.

8. The bacterium according to any of the preceding claims which produces a higher amount of the L-amino acid than the bacterium not containing said gene.

9. The bacterium according to any of the preceding claims which produces L-threonine in a concentration which is at least 100 % higher than the concentration of L-threonine produced by a bacterium not containing said gene.

10. The bacterium according to any of the preceding claims which produces L-glutamic acid in a concentration which is at least 50 % higher than the concentration of L-glutamic acid produced by a bacterium not containing said gene.

11. The bacterium according to any of the preceding claims which produces L-homoserine in a concentration which is at least 25 % higher than the concentration of L-homoserine produced by a bacterium not containing said gene.

12. The bacterium having the accession number VKPM B-7821.

13. The bacterium having the accession number VKPM B-7822.

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14. A method for producing an L-amino acid comprising cultivating the bacterium according to any of the claims 1 to 13 in a medium, producing and accumulating the L-amino acid in the medium.

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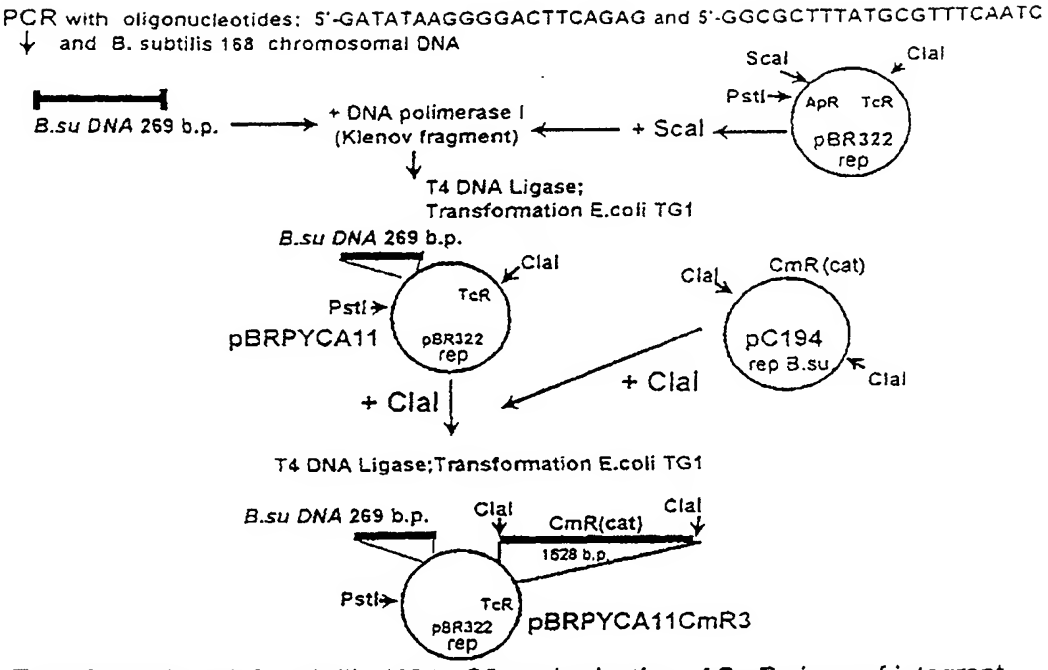

Transformation of B. subtilis 168 trpC2; and selection of CmR-clone of integrant B. subtilis trpC2 xyz(269 b.p.)::pBRPYCA11CmR3;

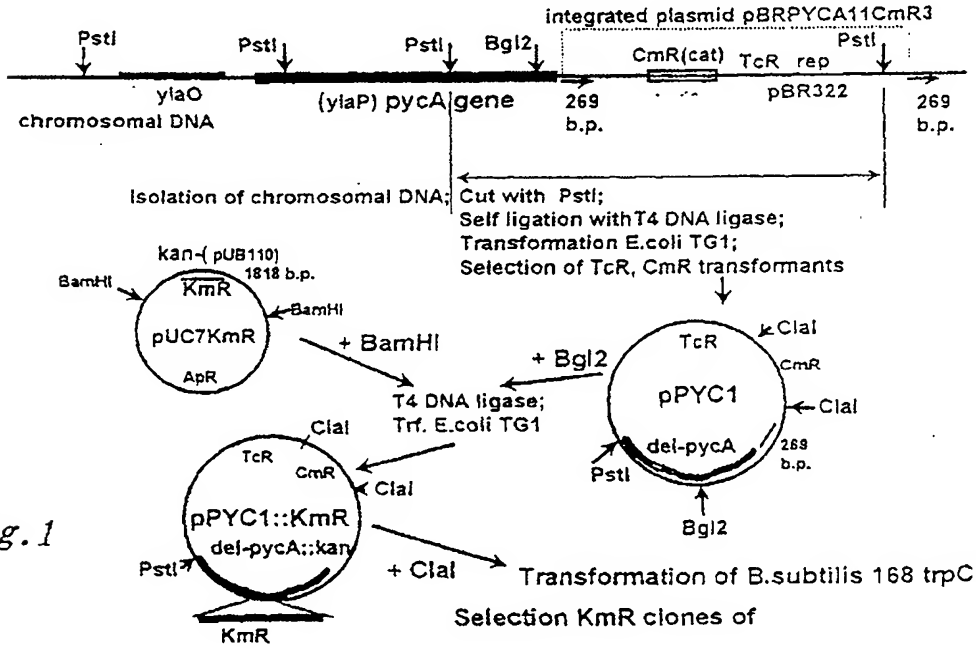

Fig. 1

B. subtilis trpC2 pycA::KmR (phenotype Asp(-) Trp(-) KmR)

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B. subtilis trpC2 pycA::KmR (phenotype Asp(-) KmR)

↓ Transduction with phages E40 lysate prepared on B. subtilis recE::tet (TcR) strain; Selection of TcR clones of

B. subtilis trpC2 pycA::KmR recE::TcR
(phenotype Asp(-) Trp(-) KmR TcR)
This strain was used as the recipient for the cloning of pycA gene.

Chromosomal DNA of B. subtilis 168 trpC2 was partially digested with EcoRI.

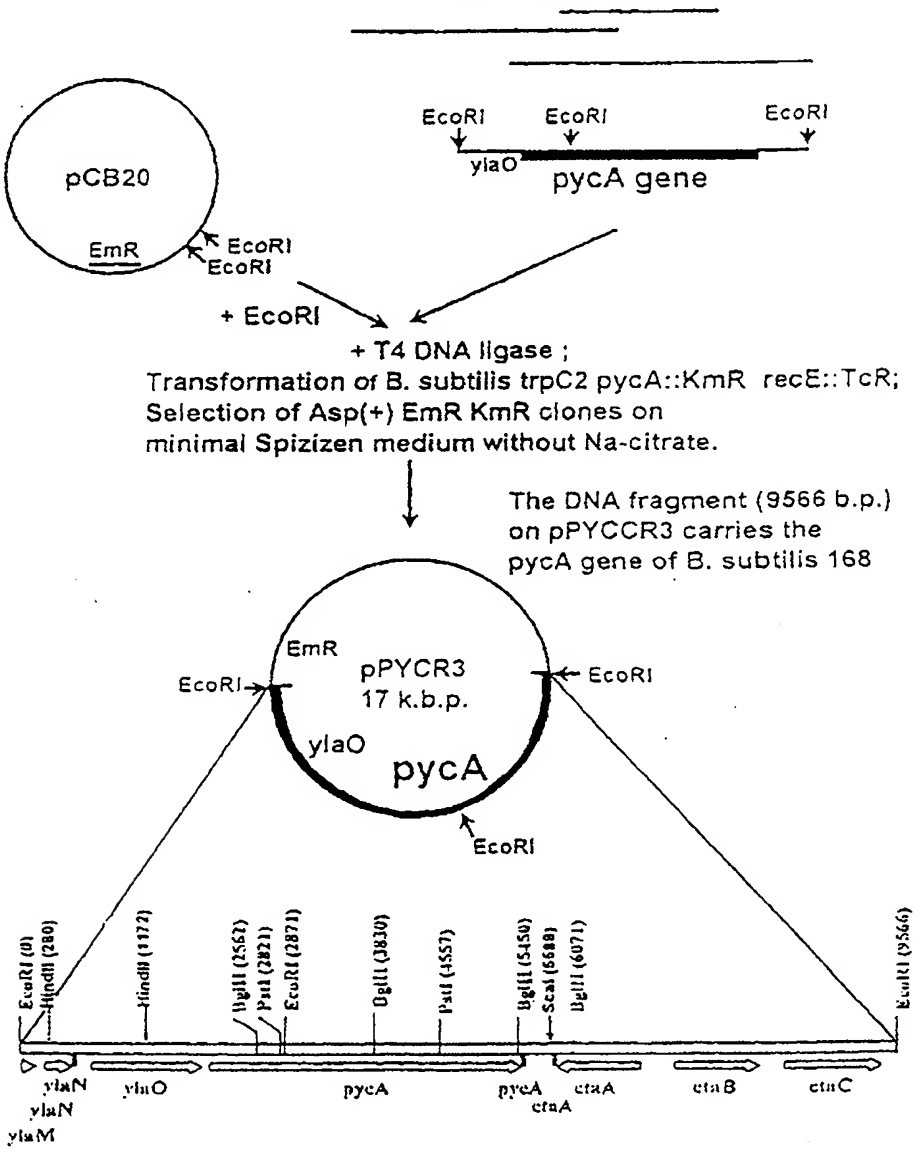

Fig. 2

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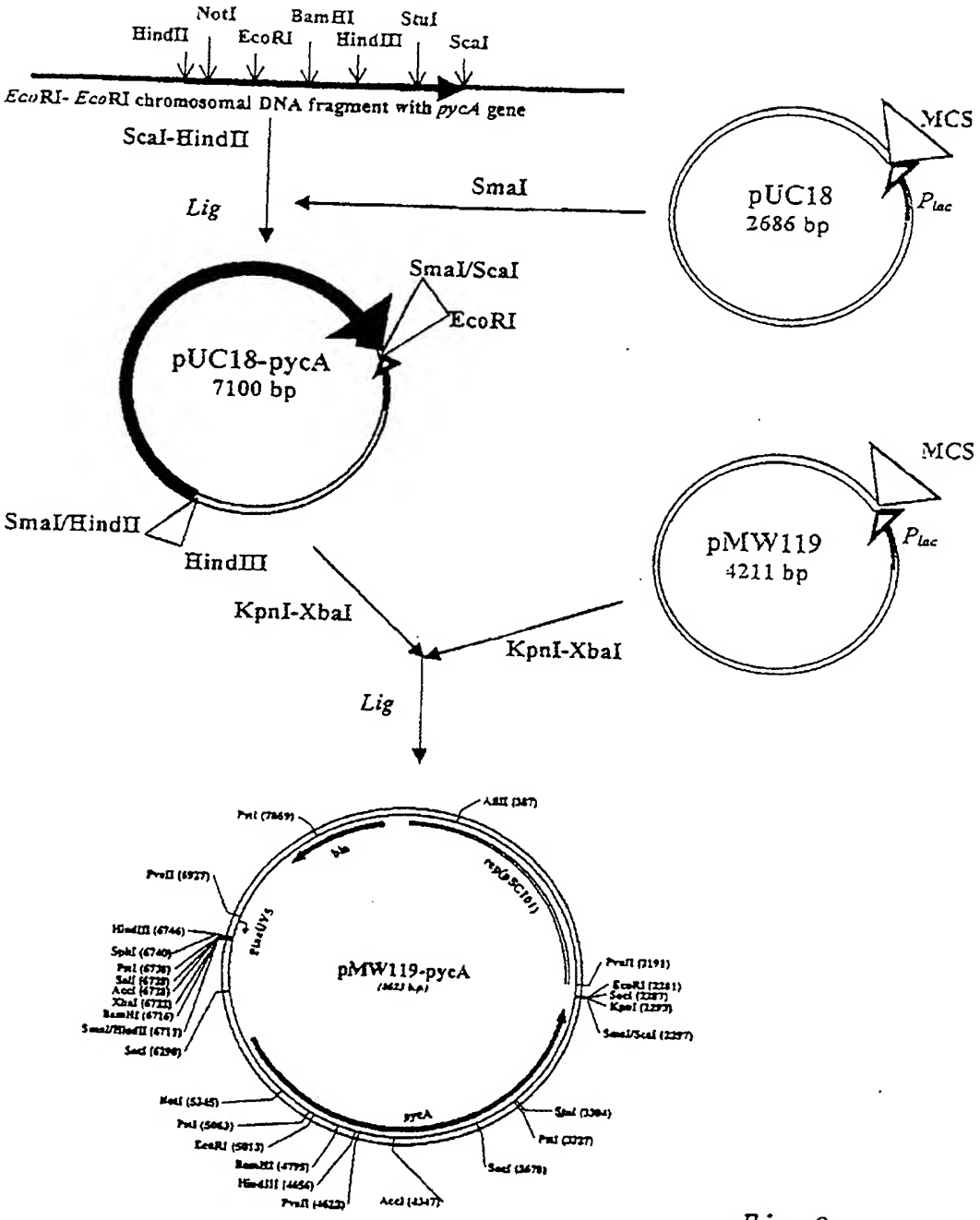

Fig. 3

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EUROPEAN SEARCH REPORT

Application Number
EP 00 12 1763

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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Y	PETERS-WENDISCH ET AL: "Pyruvate carboxylase as an anaplerotic enzyme in Corynebacterium glutamicum" MICROBIOLOGY, vol. 143, no. PART 04, April 1997 (1997-04), pages 1095-1103, XP002110209 * abstract * * page 1095, right-hand column, line 6 - page 1096, left-hand column, line 21 * * page 1100, right-hand column, line 28-47 * ---	1-14	
Y	DATABASE EMBL 'Online! EMBL; ID BSUB0008, AC Z99111, 20 November 1997 (1997-11-20) KUNST F ET AL.: "Bacillus subtilis complete genome (section 8 of 21); from 1394791 to 1603020" XP002160795 * page 36, line 28-59 * * page 85 - page 86 * --- -/--	1-14	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N C12P C12R

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 February 2001	van de Kamp, M

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 February 2001	van de Kamp, M

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